

COBALT AND GLUTATHIONE IN THE PRESERVATION OF FERTILITY AND LIFE OF SAND DOLLAR EGGS¹

OLIN RULON

*Department of Biological Sciences, Northwestern University, Evanston, Illinois, and
The Hopkins Marine Station, Pacific Grove, California*

The loss of fertility of the unfertilized egg of *Dendraster excentricus* within a few hours after removal from the ovary is of interest. During the breeding season the eggs may be 95–100% fertilizable for 24–48 hours when allowed to stand uncrowded in finger bowls of sea water at 18° C. After this interval, the capacity for fertilization decreases and the eggs soon disintegrate. When conditions are not optimum the eggs show poor fertility from the beginning. This is commonly the case with over-ripe eggs. The decay of fertility seems to be correlated with the onset and progression of the death processes which lead to disintegration.

Fertility may be prolonged or regained under certain conditions. In experiments involving the treatment of *Dendraster* eggs with lithium before fertilization (Rulon, 1946) it was found that a 1% ethyl alcohol solution would insure the fertility of the egg sufficiently to permit an adequate period of exposure to the test agent. In other experiments (Rulon, 1948) it was found that certain lots of *Arbacia* eggs, which were only 25–50% fertilizable, could be stimulated to 90–100% fertilization by treatment with Ca-free sea water for eight hours before the addition of a few drops of sperm suspension.

For a number of years the writer has been interested in modifying and controlling developmental patterns in echinoderm embryos with various enzyme inhibitors and other agents. In several cases interesting modifications have been caused by treatment of the unfertilized egg (Rulon, 1941, 1946). Cobaltous chloride was chosen for the present study because previous work (Rulon, 1956) had shown it to alter development in a definite manner when applied to the newly fertilized egg and the mid-blastula. In the work reported here it was found that cobalt had little effect on the pattern of the developing larva when only the unfertilized egg was treated. It was found, however, that cobalt possessed remarkable properties for maintaining fertility long beyond the normal period.

MATERIALS AND METHODS

The sand dollars (*D. excentricus*) were dredged from Monterey Bay during the summer of 1959 and maintained in healthy condition in tanks in the aquarium room of the Hopkins Marine Station, Pacific Grove, California. Ova were taken in large numbers from ripe females by cutting away the oral surfaces of the tests and allowing the exposed gonads to shed. Droplets of ova were drawn into medi-

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cine droppers and released in fresh sea water. After washing, the ova were placed in the various concentrations of cobaltous chloride and cobaltous-glutathione solutions for the duration of the experiments. At 24-hour intervals a number (50–100) of the eggs were removed to fresh sea water and washed. Fresh sperm suspension was added to test fertility. The chief criterion for fertilization was nuclear division. Throughout the work the eggs were kept uncrowded in covered fingerbowls out of direct sunlight and at $18 \pm 1^\circ \text{C}$. Smoking was not permitted in the laboratory since previous work by Child and others has shown that in sufficient density, tobacco smoke may cause modifications in developing embryos (Child, 1941, p. 222).

EXPERIMENTAL

I. *The effects of cobaltous chloride on fertility and cleavage*

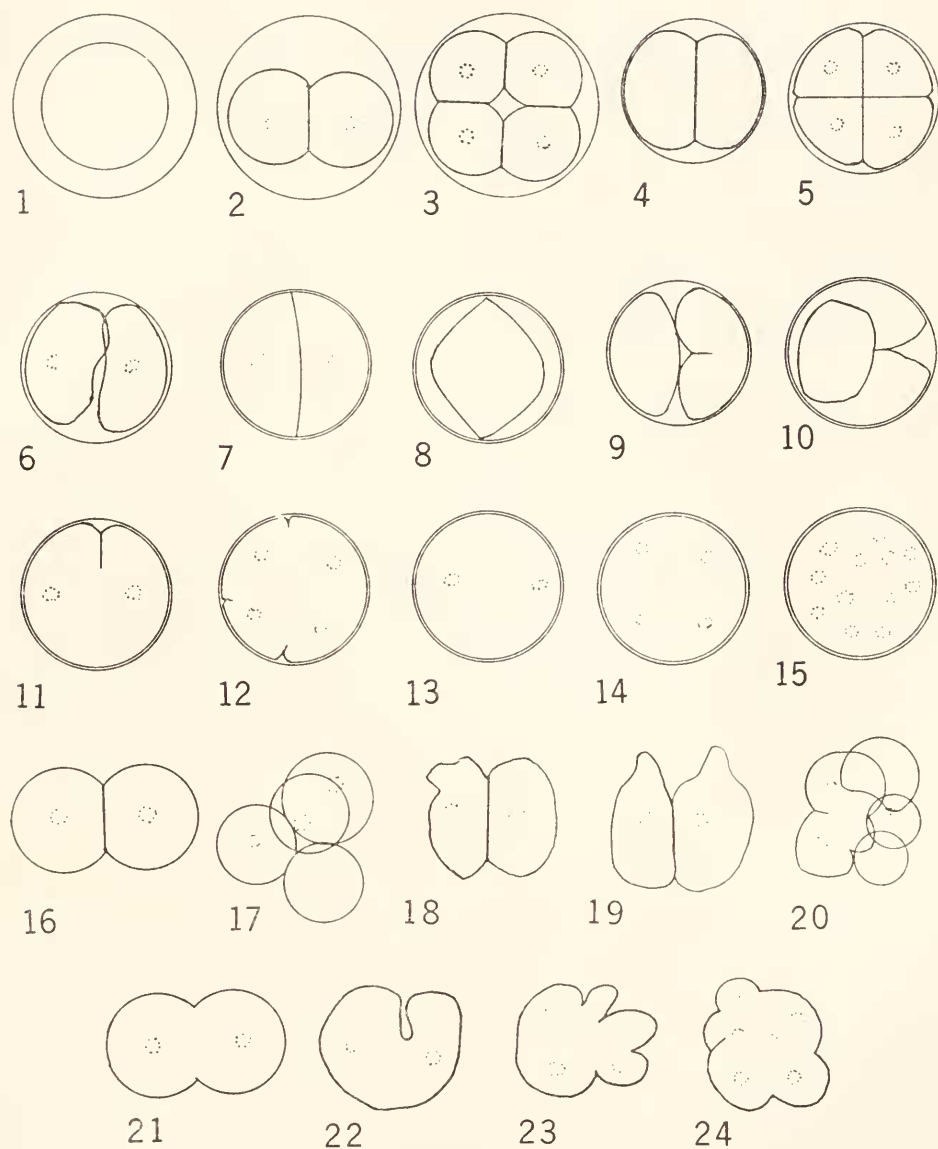
Numerous preliminary experiments indicated unfertilized eggs, in sea-water solutions of cobaltous chloride, retain their fertility long beyond those in sea-water controls. They also showed that on fertilization the cobalt-treated eggs behaved somewhat differently from the controls and that these differences were related to concentration of the cobalt and length of exposure period.

One of the most conspicuous effects of exposure to cobaltous chloride was the presence of *tight* fertilization membranes on fertilized eggs from the more concentrated solutions (compare controls, Figures 1–3, with tests, Figures 4–15). Eggs dividing in such membranes gave elongated and often incompletely separated blastomeres. If the exposure was of sufficient duration cytoplasmic division was partially or totally inhibited but nuclear division still occurred (as in Figures 11–15). If the concentration of cobalt was somewhat less but the exposure time was of long duration, the presence of a fertilization membrane could not be detected at all and cytoplasmic division graded from complete to partial (Figs. 16–24). When the blastomeres were fully cleaved they tended to become spherical and loosely attached. With long treatment many inequalities in size of cells and irregularities in cleavage patterns were to be noted.

It was soon evident that cobalt was exerting its effects largely on the cell cortex and that cytoplasmic division was more subject to inhibition than nuclear division. In general, it was found that most of the eggs that could be fertilized showed recovery in sea water and developed into plutei that were not far from normal. In some cases, however, plutei were somewhat stunted and granular but none developed in a manner comparable to those caused by post-fertilization treatment (see Rulon, 1956).

The effects of various concentrations of cobalt on the preservation of fertility are shown in Table I. Here are tabulated the results of a single series from eggs taken from one female. In this series, approximately 45% of the control eggs were fertilizable at the end of 24 hours (not in table) but none could be fertilized at 48 hours. Approximately 50% responded after treatment with $M/400$ – $M/800$ for 96 hours while approximately 25% retained fertilizability for as long as 168 hours with $M/1600$ – $M/3200$. Even in a weak solution of cobaltous chloride ($M/6400$) 50% of the eggs retained fertility for 96 hours.

These experiments definitely showed that certain concentrations of cobalt prolong the fertile life and even life itself of the egg of *Dendraster excentricus*.



FIGURES 1-24. Figure 1, newly fertilized egg of *Dendraster* with elevated fertilization membrane. Figures 2-3, normal early cleavage within fertilization membrane. Figures 4-10, eggs cleaving within tight fertilization membrane following exposure to strong ($M/400-M/1600$) solutions of cobalt. Figures 11-15, eggs with nuclear division but inhibited cytoplasmic division following long exposure to cobalt. Figures 16-24, eggs with aberrant cytoplasmic division and undetectable fertilization membranes following exposure to less concentrated solutions of cobalt.

TABLE I

The effects of different concentrations of cobaltous chloride for various intervals of time on fertilization of the eggs of Dendraster excentricus
(Values given in percentage)

| Solution used | 48 hours | 96 hours | 144 hours | 168 hours | 192 hours |
|---------------------------|----------|----------|-----------|-----------|-----------|
| Sea water (control) | — | — | — | — | — |
| M/200 cobaltous chloride | 20 | 10 | — | — | — |
| M/400 cobaltous chloride | 50 | 60 | 1— | — | — |
| M/800 cobaltous chloride | 60 | 40 | 1— | — | — |
| M/1600 cobaltous chloride | 80 | 50 | 40 | 30 | 1— |
| M/3200 cobaltous chloride | 80 | 50 | 25 | 25 | — |
| M/6400 cobaltous chloride | 80 | 50 | — | — | — |

II. *The effects of cobaltous glutathione on fertility and cleavage*

It has been thought that cobalt causes much of its effect by the fixation of thiol ($-SH$) groups (see Marston, 1952). Zinc, which has much the same action as cobalt on differentiation but at much lower concentration, appears, also, to unite with sulfhydryl (Rulon, 1955). Since the effect of zinc may be prevented with glutathione, it was suggested that this agent may well inhibit the effect of cobalt on the preservation of fertility. Accordingly, tests were run in which eggs were placed in solutions of cobaltous-glutathione in which the concentration of cobalt varied but that of glutathione was kept constant at 0.025% (a concentration which had no detectable effect by itself on the unfertilized egg). It seemed apparent that there was actual chemical union of these two substances in that clear pink solution of cobalt becomes a clear brown when the colorless glutathione was added.

TABLE II

The effects of cobaltous chloride and glutathione for various intervals of time on fertilization of the eggs of Dendraster excentricus
(Values given in percentage)

| Solution used | 48 hours | 96 hours | 168 hours | 216 hours | 312 hours |
|--|----------|----------|-----------|-----------|-----------|
| Sea water (control) | — | — | — | — | — |
| 0.025% glutathione | — | — | — | — | — |
| 50 cc. M/100 cobaltous chloride plus 50 cc. 0.05% glutathione | 95 | 95 | 95 | 90 | — |
| 50 cc. M/200 cobaltous chloride plus 50 cc. 0.05% glutathione | 95 | 95 | 90 | 70 | 50* |
| 50 cc. M/400 cobaltous chloride plus 50 cc. 0.05% glutathione | 95 | 90 | 75 | 75 | — |
| 50 cc. M/800 cobaltous chloride plus 50 cc. 0.05% glutathione | 90 | — | — | — | — |
| 50 cc. M/1600 cobaltous chloride plus 50 cc. 0.05% glutathione | — | — | — | — | — |
| 50 cc. M/3200 cobaltous chloride plus 50 cc. 0.05% glutathione | — | — | — | — | — |

* Different series of experiments.

The results of this series of experiments are tabulated in Table II where it is shown that glutathione prevented the fertility-preserving effects of cobalt only at the lower concentrations. In the stronger solutions (50 cc. $M/100$ – $M/400$ cobaltous chloride plus 50 cc. of 0.05% glutathione) cobaltous-glutathione was a far more effective agent in prolonging the fertile life of the eggs of *Dendraster* than was cobalt alone. It will be noted that the eggs are still highly fertile after 216 hours in these solutions and that in another experiment there was 50% fertility after 13 days.

It was of interest to note (data not shown in table) that while with the most concentrated solution (50 cc. of $M/100$ cobalt plus 50 cc. of 0.05% glutathione) 90% of the eggs were fertile, only nuclear division resulted. In treatment with next strongest solution, where 70% of the eggs were fertile, 30% showed only nuclear division while the remaining 40% showed some cytoplasmic cleavage as well. The 75% fertile eggs from the next solution (50 cc. $M/400$ cobalt plus 50 cc. of 0.05% glutathione) showed cytoplasmic along with nuclear division.

DISCUSSION

It does not seem likely that the effects of cobalt in the concentrations used in these experiments were due to osmotic factors. Although measurements of egg size were not made, the test eggs, before fertilization, appeared little different from the controls. In previous work by the author and many others, agents far more concentrated (*i.e.*, lithium) have been used with only slight osmotic effects. Minor changes (of the order of the concentrations used in these experiments) in salt content of the sea water produce little or no effects. It is known, however, that as echinoderm eggs age there is an increase in permeability and viscosity (see Goldforb, Landowne and Schechter, 1937, for references). It is not known that such physical changes destroy fertility in the egg but there seems to be a correlation. It should be remembered, however, that permeability to water and ions is quite different from permeability to (or penetration by) spermatozoa.

The failure of the fertilization membrane to become elevated after treatment with cobalt is difficult to explain. Tight membranes occur under a variety of conditions in these forms (*i.e.*, aging itself will cause them). It may be that long exposure to cobalt partially denatures the vitelline membrane or it may be that cobalt prevents the breakdown of the cortical granules. Certainly the subject deserves further study.

Any explanation of the mechanisms for the increase or preservation of fertility is not easy. To this worker it long has been a question as to why an ovum, containing so much in the way of nutrients, should lose its capacity to be fertilized so early. Other investigators have dealt with the problem in other eggs. The fertile life of *Arbacia* eggs may be prolonged in low-calcium sea water (Schechter, 1937). Perhaps such procedure prevents the release of calcium from its protein binding in the cortex for internal clotting (Heilbrunn, 1943, pp. 88–89) and thereby prevents or slows down processes concerned with loss of fertility and death. The fertilizable life of *Urechis* eggs may be extended with 1% alcohol or dextrose (Whitaker, 1937). It was suggested that these agents are effective by furnishing nourishment to the egg or by decreasing permeability of the egg surface. Runn-

ström (1952) reports the use of various amino acids and proteins to improve fertilization (but not to preserve fertility) in echinoderm eggs.

Of the several hypotheses given in the past it seems that the present work with cobalt may lend itself to an interpretation that is similar, in part, to one proposed by Loeb (1912, p. 155) who found that the fertilizable life of the starfish egg could be extended by cyanide or oxygen lack. He concluded that the egg becomes unfertilizable, in the normal course of events, after a damaging amount of aerobic oxidation takes place and that such agents as potassium cyanide preserved fertility by slowing down this action.

The writer has come to a tentative explanation as to the action of cobalt. This substance is well known because of its action in inducing polycythemia, possibly as the result of physiological compensation to partial anoxia, caused by the fixation of $-SH$ groups (see Marston, 1952). It is suggested that the cortex and surface of the newly shed egg are abundant in such groupings. It is well known that essential $-SH$ groups on an enzyme may be rapidly oxidized by atmospheric oxygen (see Singer, 1945). It seems not unreasonable to assume that under normal aerobic conditions in sea water, labile or soluble sulphhydryl compounds may be expected to unite with each other by oxidation to disulfide. In other words, simple proteins are united into large stable, insoluble protein chains in which the union of the individual members is through $-S-S-$ bonding (see Jensen, 1959). Such a change at the egg surface may well account for the loss in fertility in aging eggs.

It is believed that cobalt prevents this chain reaction by uniting with $-SH$ and in this manner serves in preventing the decay of fertilizability. This, indeed, seems to be the case as is shown in the experiments in which glutathione is added to the cobaltous chloride solutions. Concentrations of cobalt that were highly effective in prolonging fertility ($M/1600-M/3200$) were rendered partly or entirely ineffective by the addition of 0.025% glutathione. This shows the affinity of the two substances and supports the suggestion that cobalt unites with thiol groupings at the egg surface in the preservation of its integrity. But with more concentrated solutions of cobaltous-glutathione *the preservation of fertility was greatly enhanced!*

It is apparent that a different situation has now developed. The combination gives more protection to the egg surface and cortex than does the cobaltous ion alone. In the adsorption of cobaltous-glutathione, changes (oxidation of $-SH$) with the loss of fertility are considerably less than with cobalt alone. Such an explanation seems highly speculative until one considers the more recent work with Co^{60} Vit. B_{12} in which the combining power of this important substance with the serum proteins (alpha and beta globulins) and the protein of cerebro-spinal fluid has been so aptly demonstrated (see Meyer *et al.*, 1959). It seems quite reasonable to assume that the remarkable preserving action of cobaltous-glutathione is brought about by its union with the substances which would otherwise gradually change as fertilizability is lost.

SUMMARY

1. A range ($M/200-M/6400$) of cobaltous chloride in sea water increases the fertile life of *Dendraster* eggs. The most effective concentrations ($M/1600-$

M/3200) prolong fertilizability for over 168 hours in 25–30% of the eggs tested. None of the control eggs is fertile at 48 hours.

2. The exposure of unfertilized eggs for long periods or to high concentrations leads to tight fertilization membranes and gradual suppression of cytoplasmic division when the eggs are finally fertilized.

3. Glutathione (0.025%) prevented the fertility-preserving action of cobaltous chloride in lower concentrations but greatly enhanced it in higher concentrations. One mixture (50 cc. of 0.05% glutathione plus 50 cc. M/200 cobaltous chloride) preserved fertility in 50% of the eggs for 312 hours.

4. The work suggests that cobalt and cobaltous-glutathione unite with R-SH at the egg surface (each in a somewhat different manner) preventing oxidation and a deterioration of fertilizability.

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